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14. ABSTRACT
In this project, we are studying the role of D32G-mutant beta-catenin in the expression of secretory genes by prostate cancer cells. We believe that D32G-mutant beta-catenin potently activates a subset of beta-catenin/wnt downstream target genes, thus providing a tool for identifying "bone progression" factors activated by this pathway in prostate cancer. Results from the studies performed during this period indicate that activation of beta-catenin in prostate cancer cells stimulates a subset of beta-catenin target genes and suggest that beta-catenin expression in prostate cancer cells mediates the prostate cancer–induced new bone formation in vitro and in vivo. These results provide confidence that our gene-expression studies will be informative for identifying the beta-catenin downstream target genes that mediate the osteoblastic phenotype induced by prostate cancer cells. Immunohistochemical studies performed in human bone metastases of prostate cancer identified 4 groups based on beta-catenin intracellular distribution and expression and androgen receptor expression. It will be interesting to assess how the selected factors induced by beta-catenin in prostate cancer cells are expressed in the different groups. Although these results would only be correlative, they would provide the basis for prioritization in future studies.

15. SUBJECT TERMS
Beta-catenin, Prostate cancer, Bone metastases, Prostate cancer-bone interaction

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ANNUAL REPORT: Beta-catenin/TCF signaling and castrate-resistant progression of osteoblastic bone metastases

INTRODUCTION

In this project, we are studying the role of D32G-mutant beta-catenin in the expression of secretory genes in prostate cancer cells. Our goal is to assess the paracrine effects of these secretory genes in prostate cancer–induced osteoblast proliferation and new bone formation and how this in turn affects prostate cancer growth. The overall goal is to understand the mechanism underlying the growth of prostate cancer cells in bone during human prostate cancer bone metastases.

BODY

Task 1. At the time of the last progress report, our study results had suggested that activation of beta-catenin in prostate cancer cells stimulates a subset of beta-catenin target genes, and that beta-catenin at least partially mediates the prostate cancer–induced osteoblast activation. Studies to understand the role of the androgen receptor (AR) in this interaction are ongoing.

Task 2. The objective of this task is to determine the effect of expressing the D32G-mutant beta-catenin in prostate cancer on the growth of those cells in bone as well as to determine the bone's reaction to the presence of those cells in an androgen-depleted environment *in vivo*. For these experiments, we will use MDA PCa 118 cells with silenced beta-catenin. We will not use PC-3 cells overexpressing D32G-mutant beta-catenin because (as stated in the last progress report) we could not produce stable clones of PC-3 cells overexpressing D32G-mutant beta-catenin.

Task 2.a. was completed during the last funding period and reported on in the 2009 progress report.

Task 2.b. Inject MDA PCa 118 control cells and cells with silenced beta-catenin into the femurs of SCID mice.

Task 2.c. Obtain and analyze x-ray, magnetic resonance, and micro-computed tomography images and bone histomorphometric measures (bone mass and osteoclast numbers).

By following methods previously established and outlined in the 2009 progress report, we performed transient transfections with siRNA into MDA PCa 118b cells by using a Nucleofector kit (Amaxa; Lonza Cologne AG, Cologne, Germany). Then we injected siRNA-transfected cells into the bones of SCID mice within 24 to 48 hrs of the transfections. Initial x-ray analysis of the first successful tumor development 6 weeks after injection of MDA PCa 118 cells transfected with si-bcat or si-control suggested that bone mass increases in the bones injected with MDA PCa 118 cells transfected with si-controls but not in those injected with si-bcat-MDA PCa 118 cells (Fig. 1). We then killed those mice and analyzed the tumor-bearing bones by using bone histomorphometry. Results from these studies confirm the findings of x-ray analysis (Fig. 1). We then repeated this study by injecting MDA PCa 118 cells transfected with si-bcat or si-control into the femur of male SCID mice and killed the mice 2 weeks after cell injection. Analysis of the tumor-bearing bones by bone histomorphometry (von Kossa staining) showed that bone volume/tissue volume was significantly higher in the bones bearing MDA PCa 118b cells transfected with si-controls than in the bones bearing MDA PCa 118b transfected with si-bcat.

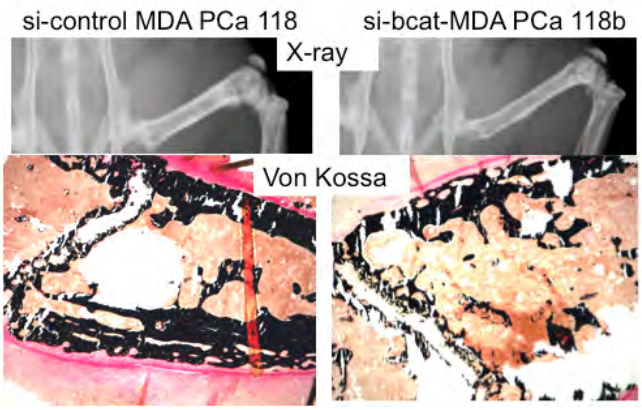

Fig. 1. X-ray anaysis and von Kossa staining of MDA PCa 118b cells transfected with si-bcat or si-controls and growing in the bones of immunodeficient mice.

Although these results suggested that silencing beta-catenin indeed reduces the prostate cancer–induced bone reaction, the reproducibility of the method is a problem. Possibly this is because the reduction in beta-catenin expression was not sufficient to consistently achieve a reduction in the prostate cancer–induced bone reaction. We initially adopted the method of transient transfections with siRNA because, in our hands, this method yielded higher efficiency than using lentiviral particles to knock down beta-catenin in MDA PCa 118 cells. However, as outlined in the previous paragraph, the results that we obtained with transient transfections were not reproducible. Thus, we will explore again the use of direct infection with lentivirus as an approach to reduce beta-catenin expression in MDA PCa 118b cells. We have a possible explanation for the unusually low infection efficiency obtained with lentivirus particles in MDA PCa 18b cells in our studies reported in the 2009 progress report. We hypothesize that MDA PCa 118b cells infected with lentivirus silence the CMV promoter that drives the expression of shRNA. To test this hypothesis, we will use SHC003V turboGFP control transduction particles (Sigma-Aldrich; St. Louis, MO) followed by puromycin selection. If the cells survive but do not express GFP, then we will conclude that the cells silence the promoter. This will mean that we are actually getting a higher infection rate than we initially thought, but the promoter is silenced. Thus, we will consider using the ubiquitin promoter to drive GFP expression. If the promoter is not silenced, and indeed we have poor infection efficiency, we will perform FACS-based selection of GFP-expressing cells.

Once we identify the problem, we will use 2 shRNA-expressing lentiviral particles—sh1-bcat (TRCN0000003845) and sh2-bcat (TRCN0000003844) (both from Sigma-Aldrich)—to knock down expression of the *beta-catenin* gene in MDA PCa 118 cells. Then we will proceed with the *in vivo* study outlined in this task (Task 2).

Task 3. Identify genes induced by the D32G- mutant beta-catenin in prostate cancer cells that mediate osteoblast activation and are expressed in human prostate cancer tissue specimens.

Task 3.a. Identify genes whose expression is regulated by the D32G mutant in prostate cancer cells. For these experiments, because we could not generate the PC-3 clones in Task 1.a. (2009 progress report), we will again use MDA PCa 118 cells with silenced beta-catenin and controls to identify genes regulated by D32G mutant.

We have performed a comparative gene-array analysis (HuGene 1.0 ST; Affymetrix, Santa Clara, CA) between MDA PCa 118 cells with silenced beta-catenin and controls. For this, we used a control group with four replicates. For beta-catenin silencing, we used siRNA (s438, Applied Biosystems/Ambion, Austin, TX) beta-cat-si1 (four replicates). We also used a second validated siRNA (beta-cat-si2 [three replicates]). Initial analysis demonstrated that the arrays are highly consistent between replicates both within treatments and between treatments. Paired *t*-test analysis did not identify any genes expressed differently between the groups of control vs beta-cat-si1 or control vs beta-cat-si2. However, mixed linear model analysis identified 10 genes expressed differently between the groups of control vs beta-ca-si1 at the false discovery rate (FDR) 0.05 (Fig. 2). These genes were *Axin2*, *APC*, and *CTNNB1* (intracellular beta-catenin complex (1)); fibroblast growth factor 19 (*FGF19*, a high-affinity ligand for FGFR4 (2)); *NOTUM*, *WNT11*, *WISP1* (regulator [*NOTUM (3)*] and mediators [*WNT11*, *WISP1 (1)*] of Wnt paracrine signals); *LEF1* (a downstream target gene and intracellular mediator of wnt canonical signals (4-6)); hyaluronan (HA) synthases 2 and 3 (synthesize HA, a core component of the extracellular matrix [ECM](7)). From these 10 differently regulated genes, we initially selected *FGF19*, *NOTUM*, *WNT11*, HA synthase 3, and HA synthase 2 and assessed their expression in MDA PCa 118 cells transfected with beta-cat-si1 and controls. We selected these genes

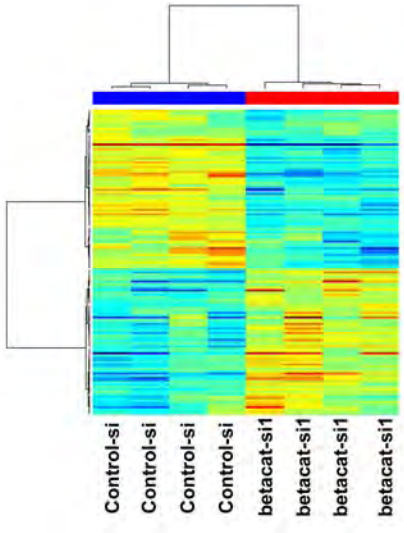

Fig. 2. Heat map of the genes expressed differently between beta-cat-si1 and control at the FDR 0.05 . This plot contains only the expression from control and beta-cat-si1.

because they are soluble factors involved in paracrine regulation of cells or are components of the ECM (Table 1). We confirmed that both beta-catenin and *FGF19* were reduced by about 3.8 times.

Table 1. Gene expression in MDA PCa 118 cells with reduced beta-catenin expression and in controls, as assessed by real-time reverse-transcription polymerase chain reaction

Gene name	Relative mean mRNA levels (x 10^4 of GAPDH)		Reduction in si-bcat cells	Function
	Control	Si-beta-cat		
CTNNB1	410.00	107.00	3.8 times	Evidence of silencing of beta-catenin expression
FGF19	410	107	3.8 times	High-afinity ligand for FGFR4
NOTUM	1146	390	2.9 times	Regulator (*NOTUM*) and mediator (*WNT11*) of Wnt paracrine signals
WNT11	260	104	2.5 times	
HA synthase 3	967.00	420.00	2.3 times	Synthesizes HA, a core component of the ECM
HA synthase 2	1040	443	2.3 times	

In addition, Lef1 is a downstream target and mediator of the wnt canonical pathway (4-6). Lef1 expression correlates with clinical stage of human prostate cancer in gene-expression profiling (correlation, 0.904; Oncomine) (Fig. 3). Further, in a comparative gene-array analysis (Affymetrix) between prostate cancer cells derived from bone metastases (n = 6) and normal prostate epithelial cells (n = 6) (all obtained by laser capture microdissection), we found that cDNA levels of Lef1 were 6-fold higher in cells derived from bone metastases. Taken together, this evidence supports the clinical relevance of this pathway.

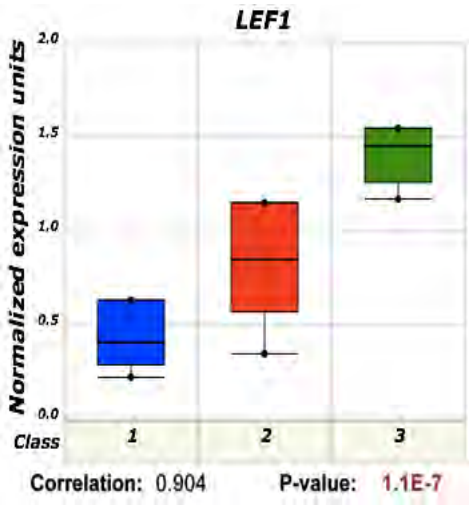

Fig. 3. Box plot of Lef1 expression in, from left to right, benign prostate (n = 6), prostate cancer (n = 7), and hormone-refractory metastatic prostate carcinoma (n = 6). Taken from Oncomine Research Platform at http://www.oncomine.org/, Varambally study.

Thus we decided to perform further screening of *FGF19* and *LEF1* expression in prostate cancer to assess the clinical relevance of these targets. With that purpose, we first established working conditions for immunohistochemical analysis while assessing expression of *FGF19* and *LEF1* in a tissue microarray containing prostate cancer xenografts derived from *adenocarcinomas* (n = 10: 4 bone metastases, 1 lymph node metastasis, 4 primary prostate cancers, and 1 soft tissue metastasis) and *small cell carcinomas* (n = 3 primary prostate cancers). We found that immunostaining with FGF19 did not produce clear results; staining had a high noise/signal ratio, which made it difficult to identify true positive expression of FGF19. On the other hand, Lef1 immunostaining was clean, with a very low noise/signal ratio.

Task 3.b. Study the role of secretory factors identified in Task 1.a. in the osteoblast activation induced by prostate cancer cells.

- Identify secreted factors that are downstream targets of D32G-mutant beta-catenin.
- Silence these factors with lentivirus vectors expressing shRNA (Mission RNAi) and perform the same experiments as described in Task 1.a. Because we could not generate PC-3 clones in Task 1.a. (2009 progress report), we will use the MDA PCa 118b cells and the factors identified in Task 3.a.

On the basis of the evidence of Lef1 expression in bone metastases cases outlined above, we will proceed with silencing Lef1 expression in MDA Pca 118b cells. Although Lef1 was previously identified as a beta-catenin target gene, it was never implicated in the prostate cancer–induced bone reaction.

Task 3.c. Study the expression of secretory factors (identified in Task 1.a.) in human prostate cancer tissue specimens (5 normal prostate, 20 primary prostate cancer, and 20 bone metastases from prostate cancer); months 24–36.

Assess expression of AR and beta-catenin and screen for mutations in the coding region of beta-catenin gene.

We previously assessed the expression of beta-catenin cytoplasmic and/or nuclear or membranous accumulation and AR expression in 10 cases of bone metastases from castrate-resistant human prostate cancers. We performed these immunohistochemical studies in consecutive formalin-fixed, paraffin-embedded tissue sections. We have now expanded that study to include expression in a total of 28 bone metastasis specimens to study the expression of beta-catenin cytoplasmic and/or nuclear or membranous accumulation and AR and Lef1 expression. We found that about 10% of prostate cancer bone metastases express Lef1 (Table 2). The cases of metastases that were positive for Lef1 expression were also positive for beta-catenin nuclear localization. This suggests that there may be a subpopulation of cases with nuclear beta-catenin/Lef1 expression, which may be associated with a clinical phenotype. We are in the process of assessing this possibility.

Table 2. Beta-catenin staining and cellular localization and AR and LEF1 immunostaining of tissue specimens derived from human prostate cancer bone metastases

Staining and localization	Percentage of cells staining positively	Number of cases staining positively (/total number of cases)
Beta-catenin membranous	20–100	27/27
Beta-catenin nuclear	20–90	9/28
Beta-catenin cytoplasmic	60–100	26/28
AR	95–100	25/28
Lef1	20–50	3/28

KEY RESEARCH ACCOMPLISHMENTS

- Our studies suggest that beta-catenin mediates the MDA PCa 118–induced increased bone mass.
- We successfully performed gene-array analysis of beta-catenin silencing in MDA PCa 118 cells and controls.
- Our studies identified a set of genes that are regulated by beta-catenin in prostate cancer cells.
- Initial studies of beta-catenin and Lef1 in human bone metastases of prostate cancer suggest that nuclear expression of beta-catenin/Lef1 may be associated with a clinical phenotype.

REPORTABLE OUTCOMES

Principal Investigator (under multiple P.I. mechanism). Beta-Catenin and Prostate Cancer Bone Metastases. R01-CA134769-01A1, NIH/NCI; priority score, 39.

Principal Investigator. FGF and Beta-Catenin/Lef1 Signaling in Prostate Cancer Bone Metastases. PC093112 – RECOMMENDED AS AN ALTERNATE. Fiscal Year 2009 (FY09) Department of Defense (DOD) Prostate Cancer Research Program (PCRP) Idea Development Award.

CONCLUSION

Our *in vitro* and *in vivo* studies *support our hypothesis that beta-catenin/TCF signaling in prostate cancer cells mediates the prostate cancer–induced new bone formation.* These results provide confidence that our gene-expression studies will be informative and constitute a good tool for identifying beta-catenin downstream target genes that mediate the osteoblastic phenotype induced by prostate cancer cells. Furthermore, our immunohistochemical studies in human bone metastases of prostate cancer (Table 2) have identified Lef1 as an important biomarker. We expect to assess the functional relevance of Lef1 in Task 3.b., and we also expect to identify additional beta-catenin downstream target genes that may help us to understand the progression of the disease.

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